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ACCELERATED COMMUNICATION

Do Canrenone and 6,7-Dihydroxylated Derivatives Compete with Ouabain at the Same Site on Na,K-ATPase?

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SUMMARY

Canrenone is the major metabolic product of the synthetic steroids spironolactone and K^+-canrenoate, used in antihypertensive therapy. Canrenone can competitively displace [^{3}H]ouabain from Na,K-ATPase [Na^+- and K^+-activated, Mg^{2+}-dependent adenosine triphosphatase (E.C.3.6.1.3)] and partially inhibit enzymatic activity. These features have led to a hypothesis that competition between canrenone and endogenous digitalis-like materials, suggested to be involved in etiology of essential hypertension, could underly the antihypertensive effect of canrenone. Surprisingly, three derivatives of canrenone (6β,7α-, 6β,7β-, and 6α,7α-dihydroxy-6,7-dihydrocanrenone) reportedly occur in normal human and rat urine. This paper characterizes the interactions with partially purified renal Na,K-ATPase of canrenone, the three 6,7-dihydroxylated derivatives, and one epoxide intermediate, synthesized from K^+-canrenoate. Canrenone and all the 6,7-substituted derivatives partially inhibited Na,K-ATPase activity (39–45%), with approximately the same apparent affinity, 100–200 μM. Canrenone displaced [^{3}H]ouabain in an apparently competitive fashion (K_i = 100–300 μM) but none of the tested derivatives significantly displaced ouabain even at very high concentrations. The ability to differentiate the ATPase inhibition and [^{3}H]ouabain displacement by modification of the 6,7-double bond indicates that inhibition of ATPase activity does not occur from within the ouabain binding site. We suggest that neither canrenone nor the 6,7-derivatives bind to the ouabain site, but rather interact with it 'allosterically.' Our findings do not support the proposed mechanisms for the antihypertensive action of canrenone.

In the late 1950s Cella *et al.* (1) and Kagawa *et al.* (2) described the structure-activity relations of a series of synthetic steroidal spirolactones with aldosterone blocking activity. Hundreds of related compounds have now been synthesized and tested for biological activity. Some of these are used in therapeutics as K-sparing diuretics and antihypertensive agents (3). Canrenone is formed *in vivo*, by dethioacetylation of spironolactone or by enzymatic lactonization of K^+-canrenoate, a water-soluble derivative.

In addition to the antimineralocorticoid and antihypertensive activity, canrenone (administered as K^+-canrenoate) is known to protect against digitalis toxicity (4, 5). Finotti and Palatini (6) found that canrenone interacts with the ouabain-sensitive Na,K-ATPase, competitively antagonizing the binding of [^{3}H]ouabain and partially inhibiting the enzyme activity. Following this report, Garay and co-workers (7) showed that canrenone partially inhibits the Na,K pump in human erythrocytes but, in cells treated with relatively high concentrations of cardiac glycosides, canrenone is actually able to partially restimulate the blocked pumps. Similar effects were also observed on macrophages (8). On the basis of this finding it was proposed (7) that the antihypertensive and other pharmacological effects of canrenone might be explained, at least in part, by antagonism with circulating endogenous digitalis-like materials suggested to be involved in the generation of essential hypertension (9, 10). Subsequently, it was proposed that this mechanism explains the antihypertensive effect of canrenone in rats with reduced renal mass, a condition in which increased levels of endogenous ouabain-like factors are reported to be present (11).

Recently it was reported that the urine of normal untreated humans and rats contain 6,7-dihydroxylated derivatives of canrenone, and effects on urinary excretion of Na and K in rats were described (12). The existence of such compounds in normal urine is very surprising, because the spirolactone family of steroids is entirely synthetic and the configuration at C17 is opposite to that of known natural steroids (13). Nevertheless, in view of the reported interaction of canrenone with the Na,K-ATPase, it was of interest to test the effects of the hydroxylated analogues on the enzyme.

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ABBREVIATION: DHC, dihydroxy-6,7-dihydrocanrenone.

Materials and Methods

Chemicals. Canrenone, 6α,7α-epoxycanrenone, and 6β,7α-, 6β,7β-, and 6α,7α-DHC were obtained by chemical syntheses described by us elsewhere. (13a). [21,22-^{3}H]Ouabain and [γ-^{32}P]ATP were purchased from Amersham International (Buckinghamshire, England). ATP, ouabain, and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or local sources as analytical grade.

Na,K-ATPase preparation. The Na,K-ATPase from pig kidney red outer medulla was purified according to Jørgensen (14) and stored at −80° until use. Its specific activity was 15 μmol of ATP hydrolyzed/min/mg of protein. Ouabain-insensitive ATPase activity was not detected.

Na,K-ATPase assay (15). Inhibition by the canrenone derivatives was tested in a medium containing the following components (final volume, 60 μl): 30 mM histidine·HCl, pH 7.5; 3 mM $MgCl_2$; 130 mM NaCl; and 20 mM KCl. Because ethanol (3.3%) was added with the inhibitors, it was also added to the control (the activity of which was inhibited by about 3%). The reaction mixture was equilibrated for 20 min at 37° with enzyme and inhibitors. The reaction was initiated by addition of ATP (3 mM) plus [γ-^{32}P]ATP (3×10^4 cpm/tube) and incubated for 10 min at 37°. The quantity of enzyme used (0.39 μg of protein) was sufficient to hydrolyze about 15% of the substrate in the absence of inhibitors. Tubes containing enzyme but no inhibitor (control), or no inhibitor and no enzyme (blank), were also included. The reaction was stopped with 300 μl of a mixture of 5% perchloric acid and 1 mM P_i-Tris, and tubes were transferred to an ice bath. One hundred microliters of ammonium molybdate (5% in 4 N H_2SO_4) and 700 μl of 2-methyl-1-propanol were added. The tubes were shaken three times for 30 sec at 5-min intervals and centrifuged at 12,000 × *g* for 30 sec in an Eppendorf microcentrifuge. Portions (500 μl) of the organic layer were removed to counting minivials and 3 ml of scintillation fluid were added. The vials were shaken and counted. Ouabain-sensitive ATP hydrolysis was estimated from the $^{32}P_i$ released minus the value obtained for the blank.

[^{3}H]Ouabain binding assay. Binding of [^{3}H]ouabain was assayed at equilibrium, by a filtration technique, in 100-μl final volume. In condition A the reaction mixture contained the following constituents, in final concentration: 50 mM Tris·HCl buffer, pH 7.4; 80 mM NaCl; 4 mM $MgCl_2$; 2 mM ATP (Tris, vanadium-free), and 11.2 nM [^{3}H]ouabain (5×10^4 cpm/assay tube). Binding was initiated by the addition of 1 μg of enzyme suspended in 40 μl of a mixture of 25 mM histidine and 1 mM EDTA. After 60-min incubation at 37°, 3 ml of ice-cold 50 mM Tris·HCl was added and tubes were transferred to an ice bath. The suspensions were filtered on Whatman GF/B filters and these were washed twice with 3 ml of the same buffer. Scintillation fluid containing Triton X-100 was added and after 24 hr the samples were counted for radioactivity. Specific binding was calculated by substracting that observed in the presence of 1 mM unlabeled ouabain (nonspecific) from the radioactivity bound (total binding). Nonspecific binding represented up to 5% of the total binding under control conditions. Canrenone derivatives were solubilized by addition of up to 2% ethanol. These concentrations of alcohol had no effect on control and inhibition values. In some experiments (condition B), 1.5 μg of protein and 2 nM [^{3}H]ouabain were used.

Results

Na,K-ATPase inhibition by canrenone and derivatives. Canrenone, 6α,7α-epoxycanrenone and three dihydroxylated derivatives (6α,7α-, 6β,7β-, and 6β,7α-DHC) were compared for inhibitory effects on Na,K-ATPase activity of enzyme purified from pig kidney red outer medulla. Fig. 1 shows the loss of enzyme activity with increasing concentrations of 6α,7α-DHC and depicts the pattern of partial inhibition characteristic for all these derivatives. Partial inhibition was found also for canrenone in brain enzyme (6) and in human erythrocytes (7).

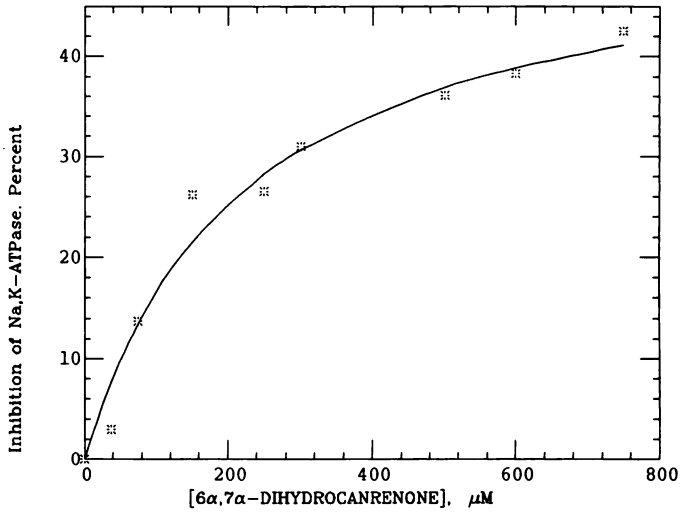

Fig. 1. Inhibition of renal Na,K-ATPase by 6α,7α-DHC.

TABLE 1

Kinetics parameters for inhibition of Na,K-ATPase by canrenone and 6,7-derivatives

Kinetic parameters (± standard error) were calculated using a personal computer and a nonlinear regression analysis for the hyperbolic inhibition curves.

Compound	Maximal Inhibition	$K_{½}$
	%	μM
Canrenone	43 ± 4.5	158 ± 43
Epoxide	41 ± 2.1	67 ± 14
6α,7α-DHC	45 ± 6.5	156 ± 47
6β,7β-DHC	39 ± 6.8	127 ± 46
6β,7α-DHC	41 ± 9.0	147 ± 60

Table 1 summarizes the data obtained for maximal per cent inhibition (maximal inhibition) and the inhibitor concentration for half-maximal inhibition ($K_{½}$) for all the compounds tested. As can be seen, the maximal level of inhibition is not significantly different for all of the compounds (varying in the range 39 to 45%). The $K_{½}$ is also essentially the same with the exception of the epoxide, which appears even somewhat lower.

Displacement of [^{3}H]ouabain by canrenone and derivatives. The ability to displace [^{3}H]ouabain bound to the renal Na,K-ATPase by canrenone and the 6β, 7β-diol is shown in Fig. 2. The other diols behave essentially as the 6β, 7β-derivative. Canrenone displaced the [^{3}H]ouabain as expected (2) in this experimental condition; however, the dihydroxylated derivative was unable to significantly displace ouabain from its binding site, even at concentrations up to 750 μM, which was the solubility limit. A comparison of the ability of canrenone and the three diol derivatives, at a single high concentration, to displace ouabain is shown in Table 2. In no case was significant displacement of ouabain produced by the dihydroxylated derivatives. The K_i for canrenone in experiments like that in Fig 2 was obtained from a linear regression analysis of the plot F_o/B_o versus F_i, assuming competition between ouabain and canrenone (Fig. 2, *inset*) where

$$\frac{F_o}{B_o} = F_i \times \frac{K_o}{(nE - B_o)\,K_i} + \frac{K_i \cdot K_o}{(nE - B_o)\,K_i}$$

and F_o = concentration of free ouabain, B_o = concentration of bound ouabain, F_i = concentration of free inhibitor, which in our conditions is effectively equal to the total concentration of

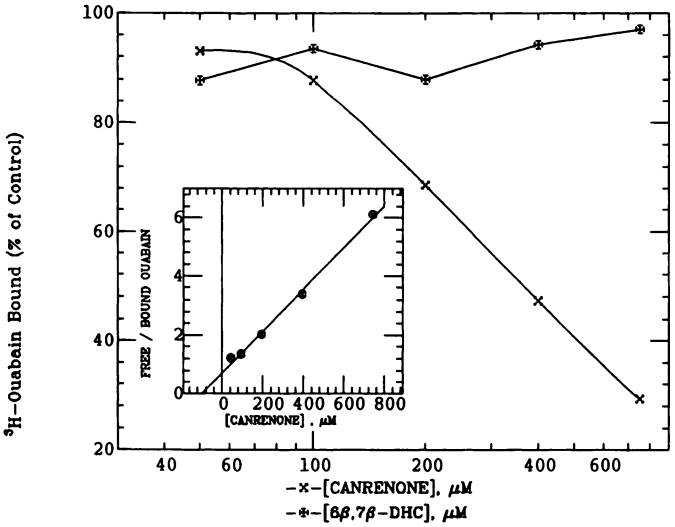

Fig. 2. Comparison of displacement of [^{3}H]ouabain by canrenone and 6β,7β-DHC. Condition B was used for this experiment. The data of the curve with canrenone were transformed to produce the linear plot in the *inset*.

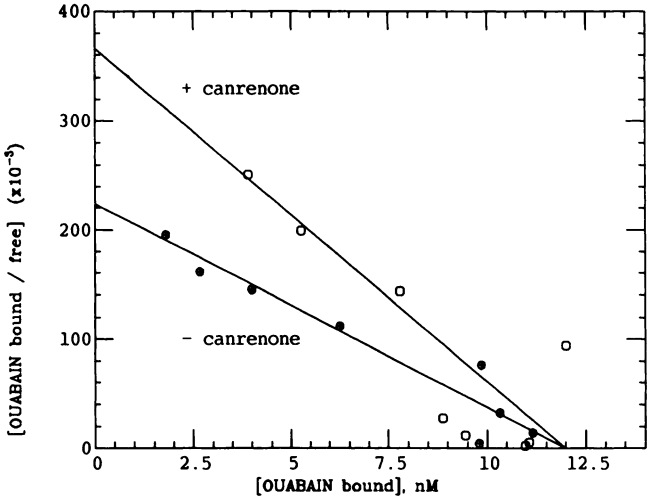

Fig. 3. Apparent competition between ouabain and canrenone. Condition A was used. Binding of [^{3}H]ouabain was measured over a range of concentration of ouabain, 11–5000 nM, in the absence (Φ) or presence (O) of canrenone, 500 μM. Slopes of the Scatchard plot were obtained by linear regression.

TABLE 2

A comparison of ouabain binding in the presence of canrenone and 6,7-DHC

Oubain binding was measured in condition A. Canrenone and derivatives were present at a fixed concentration of 500 μM. Values represent mean ± standard error of quadruplicate measurements.

Compound added	[^{3}H]Ouabain bound
	% of control
Canrenone	39.23 ± 1.14
6β,7α-DHC	91.69 ± 3.06
6β,7β-DHC	100.23 ± 1.72
6α,7α-DHC	94.88 ± 3.34

inhibitor, K_i = dissociation constant of the inhibitor, K_o = dissociation constant of ouabain, n = number of identical and independent ligand binding sites, and E = enzyme concentration. For $F_o/B_o = 0$, $-F_i = K_i$.

The fact that good linearity is observed supports the assumption of competition between ouabain and canrenone. In the experiment of Fig. 2, a K_i of 99 μM was observed. In another experiment using a total ouabain concentration of 11.2 nM (condition A in Materials and Methods) a K_i of 260 μM was calculated. The difference in K_i observed in the two experiments was not expected, on the assumption of simple competition. Notice that the range of canrenone concentrations for displacement of ouabain (K_i = 100–260 μM) is quite similar to that for inhibition of Na,K-ATPase activity (K_i = 158 ± 43 μM; Table 1).

The apparent competition between ouabain and canrenone was examined in more detail in the experiment of Fig. 3, in which we looked at displacement of [^{3}H]ouabain by increasing concentrations of unlabeled ouabain, in the absence and presence of a fixed concentration of canrenone (500 μM). The behavior observed is indeed that expected for competition and confirms that the original observation on brain microsomes holds also for the kidney enzyme. The apparent K_D values for ouabain in the absence and presence of canrenone were 32.8 and 53.6 nM, respectively, and the maximal amount of ouabain bound was 1.2 nmol/mg of protein. The ratio of the slopes in the absence and presence of canrenone, $(F_i + K_i)/F_i$, equals 1.631 in this experiment, and hence a K_i of 315 μM was calculated.

Discussion

Our results throw light on the mechanism of interaction of canrenone and derivatives with the Na,K-ATPase and on the postulated mechanism of interaction of canrenone with ouabain and the putative ouabain-like compounds. The findings of Finotti and Palatini (6), that canrenone competitively antagonizes ouabain binding and partially inhibits ATPase activity, and those of Garay and co-workers (7), demonstrating partial reactivation of ouabain-blocked Na,K pumps by canrenone in red cells, were the basis for the suggestion that competition between canrenone and the hypothetical circulating ouabain-like compound is involved in the antihypertensive effect of the drug (11, 16). It was suggested by both groups that the antagonism occurs at the digitalis receptor site, although the findings did not rule out an indirect antagonism between canrenone and ouabain.

One factor said to favour true competition is the 'structural similarity' (6). However, as shown in Fig. 4, canrenone is a quasi-planar molecule in which the butanolide ring is fixed and connected by a spiro-carbon to the D-ring of the steroid, whereas ouabain is nonplanar, with a strongly bent molecular shape due to the *cis*-junction between rings A and B, and rings C and D. Its lactonic ring is separated from C17 by an extra C—C single bond, i.e. by more than 2 Å from the steroid nucleus, and is free to rotate. Repke and co-workers (17) found that the minimal structural requirement in cardiac glycosides for specific receptor recognition is 5β,14β-androstane-3β,14β-diol. If any of these features are changed, most or all of the biological activity is lost. Repke concluded from thermodynamic considerations that canrenone is not congeneric with digitalis. Therefore, it is not obvious *a priori* that a stereospecific digitalis binding site should recognize also canrenone.

The following arguments, in ascending order of importance, from the present work suggest strongly that in fact the apparent competition between canrenone and ouabain does not occur at the same site on the protein.

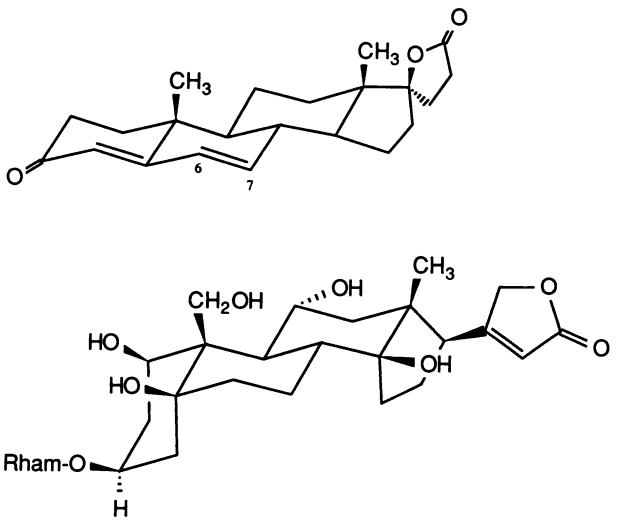

Fig. 4. Stereochemical structures of canrenone and ouabain. *Upper*, canrenone; *lower*, ouabain. Note that rings A and B in canrenone are represented in the chair conformation, although strictly the half-chair conformation is more correct. This does not affect the quasi-planar representation of the molecule.

1. Canrenone appears to compete with ouabain on the partially purified renal Na,K-ATPase, as found previously on brain enzyme (6). However the K_i for canrenone's displacement of ouabain was different when different concentrations of ouabain were used, (K_i = 260 μM with 11.2 nM ouabain and 100 μM with 2 nM ouabain). This is not expected for simple competition.

2. The K_i for canrenone in displacing ouabain, 100–260 μM, is not appreciably different from the $K_{½}$ for inhibition of ATPase activity, 158 ± 43 μM. This is quite different from the behaviour of ouabain, for which the dissociation constant for binding in optimal conditions, $K_D \simeq 2 \times 10^{-8}$ M, is about 2 orders of magnitude lower than that required for half-maximal inhibition of Na,K-ATPase in the standard reaction medium, $K_{½} \simeq 1\text{–}2 \times 10^{-6}$ M. The difference in the apparent ouabain binding affinities reflects the different preponderance of conformations of the protein with high (E_2P) or low (E_1) binding affinity in the two experimental conditions. If canrenone was a true competitor with ouabain one might expect it also to display these differences.

3. All of the 6,7-substituted canrenone derivatives used in this work show degrees of inhibition of ATPase activity entirely comparable to that of canrenone but no significant displacement of ouabain. Thus, the two effects, inhibition of ATPase and displacement of ouabain, are entirely dissociable, i.e., it is not binding to the specific ouabain site that causes inhibition of ATPase activity. Therefore, the high degree of true structural similarity between canrenone and the derivatives makes it likely also that inhibition of ATPase by canrenone does not occur from within the ouabain binding site.

What are the structural requirements for inhibition of ATPase and displacement of ouabain by the spirolactonic compounds? *In vitro*, K^+-canrenoate neither inhibits ATPase activity nor displaces ouabain (18). From this result and those in this paper it would appear that for inhibition of Na,K-ATPase activity the lactone ring must be intact, whereas for displacement of ouabain an extra requirement is that the 6,7-olefinic bond must not be substituted. The binding of spirolactones to plasma proteins (19) or to mineralocorticoid receptors (20–22) has been shown before to be sensitive to substitution at the C6 and C7 positions or to the hydrolysis of the butanolide ring. The absolute requirement for the intact lactone ring could reflect the requirement of a specific binding site, but it is equally possible that this merely reflects a reduction in polarity of the molecule (19).

In view of the evidence presented here that canrenone and ouabain do not compete directly, we must assume that the antagonism is indirect. Thus we propose that the canrenone interacts with the ATPase in a relatively nonspecific way from another site. This could be from within the lipid bilayer or from a hydrophobic pocket on the surface of the protein. If either was the case it would explain the necessity for closure of the lactonic ring, i.e., in order to increase partition into the lipophilic environment (19). In support of a relatively nonspecific interaction with the ATPase are recent findings that canrenone interacts also with other membrane transport proteins, producing, for example, inhibition of Ca-dependent K channels in macrophages (8) and activation of amiloride-sensitive Na channels in toad bladder vesicles.[1]

What is the significance of our findings for the possible antihypertensive effect of canrenone? The concept of direct competition between canrenone and ouabain made it attractive to speculate that competition between canrenone and the putative endogenous digitalis-like compound lies behind the antihypertensive action of canrenone. One problem with this hypothesis, as it stands, is that the therapeutic dose of canrenone is about 1 order of magnitude lower than the *in vitro* requirement for detectable effect on the Na,K-ATPase. Antagonism between spirolactonic drugs and digitalis compounds may well occur *in vivo* (for example, Refs. 4, 23, and 24) but the present evidence that this antagonism on the ATPase is indirect makes it much less attractive to propose a specific and true competition between canrenone and an unidentified endogenous ouabain-like compound.

Therefore the present findings do not seem to favor the proposed mechanism for the anti-hypertensive effect of canrenone. A test of the hypothesis would be to look at effects of the 6,7-dihydroxylated analogues of canrenone on blood pressure. An anti-hypertensive action would effectively rule out this hypothetical mechanism.

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